

**UNITED STATES AIR FORCE  
RESEARCH LABORATORY**

**HYPERBARIC OXYGEN MODULATES  
GENE EXPRESSION IN THE  
ISCHAEMIC GERBIL BRAIN**

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## TECHNICAL REVIEW AND APPROVAL

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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

## FOR THE DIRECTOR



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## PREFACE

This one of a series of technical reports describing the results of experimental laboratory programs conducted at AFRL/HEST. This document serves as an interim report concerning predictive toxicology methods. Interruption of blood supply (ischaemia) by wounds, surgery, or blunt trauma results in tissue injury. However, most damage occurs as blood rapidly returns (reperfusion) to the tissues. This research was conducted to better understand the mechanisms of ischaemia/reperfusion toxicity and the potential effects of hyperbaric oxygen treatment, in order to better establish the potential hazards for humans. Previous studies have demonstrated that hyperbaric oxygen treatment may suppress some of the detrimental effects of the ischaemia/reperfusion injury. The results of this study provide some of this necessary insight by describing the response of molecular biomarkers of effect, caused by exposures to hyperbaric oxygen and ischaemia/reperfusion conditions. The research described in this report began June 1996 and was completed in August 1997 under U.S. Air Force Contract No. F41624-96-C-9010 (ManTech/Geo-Centers Joint Venture). LtCol. Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, Armstrong Laboratory, Toxicology Division. Participation of Kenneth L. Hensley, PhD., and Jennifer Raker, graduate student, was supported in 1996 by the Air Force Office of Scientific Research Summer Research Program.

The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

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## LIST OF ABBREVIATIONS

HBO,h	hyperbaric oxygen
INOS	inducible nitric oxide synthase
ODC	ornithine decarboxylase
HSP	heat shock protein
I/R	ischaemia/reperfusion
DNA	deoxyribonucleic acid
ROS	reactive oxygen species
NO	nitric oxide
O <sub>2</sub> •	superoxide radical
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
PCR	polymerase chain reaction
cDNA	copy DNA
OD	optical density
SEM	standard error of the mean
S	sham
I	ischaemia

## I. INTRODUCTION

The efficacy of hyperbaric oxygen (HBO) as a therapeutic agent, especially for the treatment of stroke or other ischaemia/reperfusion (I/R) injuries, has been researched and reviewed (1, 2, 3). However, there have been no clear conclusions on this matter. At the heart of the problem is that the exact molecular mechanisms of effect of combined HBO and I/R treatments is not fully understood. In this presentation, we consider the experimental model for HBO and I/R effects on the brain.

Whereas extended periods of ischaemia alone generally lead to tissue necrosis, brain damage resulting from ischaemia/reperfusion can occur at a number of stages. Early phase damage is thought to be largely mediated by calcium influx into cells. Along with this, oxidative damage arises as a primary culprit for the ensuing degeneration of neuronal tissue after reperfusion (4, 5, 6). Endpoints for DNA damage and fragmentation (markers for apoptosis) are evident after I/R events (7, 8). In later stages of the response, neutrophils and inflammatory cytokines can participate in the subsequent cascade of events (5, 6). These cytokines can mediate the attraction of additional macrophages, activate cells, and stimulate gene expression. Nitric oxide (NO) and reactive oxygen species (ROS) are particularly cogent to macrophage- and microglia-mediated post-ischaemic events (4). Levels of both of these molecules are increased as a result of I/R events. Whereas NO may be protective or beneficial to neurons, in the presence of superoxide radical ( $O_2^{\bullet-}$ ) NO readily reacts, forming peroxynitrite species (9). Peroxynitrite is consistently found to cause cell damage and death.

The expression of certain genes is modulated in response to I/R events. In particular, the inducible form of nitric oxide synthase (iNOS) present in macrophages, glia, and vascular cells, is induced by I/R and accounts for the related increase in NO production (10). The significance of this increased NO concentration in later stage tissue damage (11, 12) is discussed above. Polyamines are another group of chemicals that play a potential role in I/R injury. These molecules can disrupt neuronal integrity and facilitate cerebral edema formation (13, 14, 15, 16). Ornithine decarboxylase (ODC), the rate-limiting enzyme for polyamine biosynthesis (from ornithine), is regulated at the transcriptional level by I/R events and the increased enzyme activity closely correlates with increased mRNA levels (17). The increased production of polyamines has been hypothesized to be in competition for arginine with NO

synthase enzymes (18). Among other genes modulated in response to I/R are the heat shock proteins (HSPs). Specifically, HSPs 27, 60, and 70/72 have exhibited increased expression due to I/R exposure. HSP70 response is elicited by many different stressors, but in particular, it is related to the development of a limited tolerance to ischaemia (19). Although this tolerance is not evidenced specifically by changes at the transcriptional level, increased amount of HSP70 mRNA remains an indicator of I/R injury (9).

The effects of hyperbaric oxygenation on gene expression varies somewhat in comparison to those of I/R injury. The ability of HBO to prevent delayed neuronal death, is a central reason to consider HBO in the context of I/R effects on gene expression (20). This preventive effect sustains, while HBO still induces mild oxidative stress (21), but without associated early brain damage (22). HBO has been shown specifically to decrease the expression of iNOS, potentially resulting in decreased amount of NO (21). The response of the ODC gene to HBO has not been previously characterized. The consideration of potential HSP70 response to HBO is confounded by previous studies, such as those reporting minimal response (20) or a response that increased with multiple HBO exposures (23).

The purpose of this study was to better define the molecular events associated with ischaemia/reperfusion injury and hyperbaric oxygen exposure by analyzing the expression of specific mRNAs of genes that responded to these treatments (iNOS, ODC, and HSP70). We intend to define whether there is regulation of ODC at the transcriptional level for HBO exposed animals and how it compares with ODC expression for animals exposed to ischaemia. We have exposed animals to combinations of ischaemia and HBO, and assessed the ability of HBO to ameliorate or enhance the response of certain genes (ODC, HSP70, and iNOS) to the ischaemic insult.

## **II. MATERIALS AND METHODS**

### **Animal Exposures and Sampling**

Male Mongolian gerbils were anesthetized with pentobarbital (40 mg/kg, i.p.) and prepared for transient global ischaemia via carotid artery occlusion as previously described by Chandler and others (See 24, 35, 26). Following surgery, animals were individually housed and allowed to recover for 2 days. Animals (5 animals per treatment group) were subjected to a single treatment scenario as described in the table below (Table 1). Ischaemia was accomplished by bilateral carotid occlusion for a period of 10 min. Animals exposed to hyperbaric oxygen (HBO, 2 atm) were placed in a pressure- and gas- regulated steel chamber for a single 1 hour exposure. The sham and HBO groups underwent the same surgical procedures as those receiving ischaemia, but without carotid ligation. Animals were sacrificed by decapitation at specific times following reperfusion (2 hrs and 24 hrs) and the hippocampus was frozen in liquid nitrogen for RNA analysis, while other portions of the brain were also frozen in liquid nitrogen for subsequent enzyme and protein analysis. Samples were then transferred to a -140°C freezer to be kept for analysis.

### **RNA Analysis**

Total RNA was extracted from brain tissue using a commercial reagent (RNA Stat 60) and dissolved in specially prepared formamide (Formazol), both from Tel-Test "B", Inc. (Friendswood, TX). Extracted RNA was stored at -140°C until Northern analysis. RNA samples were electrophoresed (40µg/lane) on 1% formaldehyde gels and transferred to positively charged nylon membranes (MAGNA-NT, Micron Separations, Inc., Westboro, MA). Membranes were then UV-crosslinked and baked at 80°C for 1hr.

The ODC cDNA probe was made from a 720bp PstI restriction fragment isolated from a plasmid clone of mouse ODC (pMK934) obtained from Dr. Rosemary Elliott (Roswell Park Memorial Institute, Buffalo, NY). The HSP70 cDNA probe (651bp) was made from a HSP70 cDNA fragment we amplified by RT-PCR from gerbil mRNA. The iNOS cDNA probe (616bp) was prepared from a PCR fragment provided by Dr. Ken Hensley (OMRF, OK). The cDNAs were labeled with [32]P-dCTP by random primer method. Hybridizations of cDNAs were performed at 42°C. After overnight hybridization, membranes were exposed to X-ray film at -70°C for 1 to 5 days. Band intensities on developed films were quantified by laser scanning

densitometry (Bio-Rad Instruments Model 670 Densitometer with Molecular Analyst Software, Version 1.3, Bio-Rad, Laboratories, Hercules, CA). Equivalent loading among gel lanes of RNA was determined by hybridization with a rat 18S rRNA probe (AMBION, Austin, TX).

**TABLE 1. Experimental design**

Time Course of Experiment →				Group	
60 min	2 hrs 40 min		Sacrifice	H	2 hrs
HBO					
	10 min	2 hrs	Sacrifice	I	
	ISCH				
60 min	30 min	10 min	2 hrs	H+I	24 hrs
HBO		ISCH			
		10 min	60 min 1 hr	I+H	
		ISCH	HBO		
60 min	24 hrs 40 min		Sacrifice	H	24 hrs
HBO					
	10 min	24 hrs	Sacrifice	I	
	ISCH				
60 min	30 min	10 min	24 hrs	H+I	
HBO		ISCH			
		10 min	60 min 23 hrs	I+H	
		ISCH	HBO		
	24 hrs		Sacrifice	S	

### Statistical Analysis

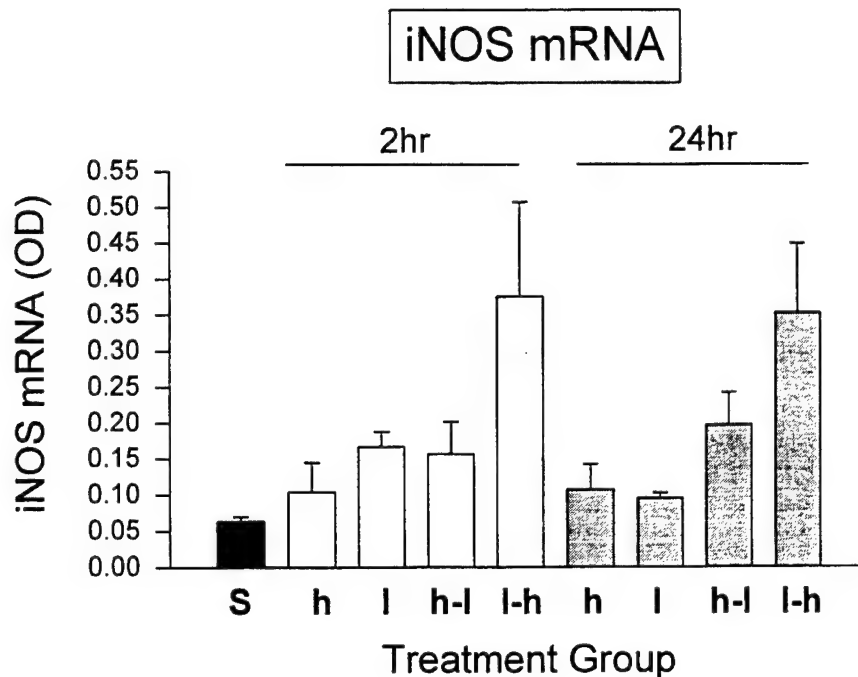
RNA levels measured by densitometry and reported in optical density (OD, mean  $\pm$  SEM) units were analyzed by one-way ANOVA with BMDP (BMDP Statistical Software, Inc., Los Angeles, CA), with multiple comparisons between groups by *t* test. Differences were identified as statistically significant at  $p \leq 0.05$ . Unexpected mortality accounts for group sizes less than  $n=5$ .

### III. RESULTS AND DISCUSSION

Expression of iNOS mRNA was detected in all samples (Fig. 1). The levels of response ranged from 140-550% that of the sham control group. Table 2 shows the statistical significance of comparisons made between groups ( $p < 0.05$ ). HBO-only and ischaemia-only groups did not have iNOS mRNA levels different from the sham. The largest elevations (almost 2 times that of the next highest level) of iNOS were seen in the groups that received post-ischaemic HBO (540-550% over sham control). Responses in most other groups were significantly lower than in those two groups. The effect of HBO before ischaemia did show an increase over the sham for both the 2hr and 24hr time points (230% and 300%, respectively). These results are not consistent with HBO decreasing available NO, in vivo, based in a decrease in iNOS expression. To the contrary, in the case of HBO exposure after an ischaemic insult, iNOS mRNA levels are markedly increased. Thus, any potential beneficial effect of HBO exposure is indicated by this specific response.

ODC mRNA levels were detected in all treatment groups (Fig. 2). Table 2 shows the statistical significance of comparisons made between groups ( $p < 0.05$ ). Levels of response as compared to that of the sham control group ranged from a decrease of 50% (HBO-only group) to an increase of 150% (2hr ischaemia-only group). The highest levels were evidenced in the ischaemia-only groups (150% at 2hr and 130% at 24hr). The effect of HBO treatment before an ischaemic exposure resulted in a significantly lower level of ODC mRNA expression at the 24hr time point (50% of sham) than the ischaemia-only group at the 24hr time point (130% of sham). When HBO was given after ischaemic insult, there was an equally elevated amount of ODC mRNA compared to the ischaemia-only group at 2hr. However, by the 24hr point, while the ischaemia-only group has an ODC mRNA level at 130% of sham, the ischaemia+HBO group has a level only 50% of the ODC expression for the sham control. These data show that ODC mRNA expression does not appear to be induced by HBO, to an extent greater than seen in the sham groups. In addition, HBO appears to suppress ischaemias induction of ODC mRNA expression. At 24hr, this is the case whether HBO precedes or follows ischaemic insult. The mechanism by which HBO elicits this response has not been strictly defined. However, the decreased levels of ODC correlate well with decreased ODC enzyme activity and polyamine production as identified elsewhere (17). Our identification of the ODC mRNA expression response to combined exposure to HBO and ischaemia has not been previously characterized.

HSP70 mRNA was detectable for all of the treatment groups (Fig. 3). The mean responses for HSP70 mRNA expression to most combinations of HBO and ischaemia range from 150-350% of the level for the sham group. However, HSP70 mRNA in only two of treatment groups (24hr ischaemia and 24hr HBO) are significantly elevated (>250%) versus the sham group. There are no apparent differences among the other treatment scenarios. HSP70 responds to a number of different chemical and physiological stressors. Previous studies of HSP70 mRNA response to HBO or I/R have not shown consistent results. Here, there is a perturbation in all of the groups, but the responses are highly variable.



**FIGURE 1.**

**Effects of HBO and/or ischaemia-reperfusion on iNOS mRNA Expression**

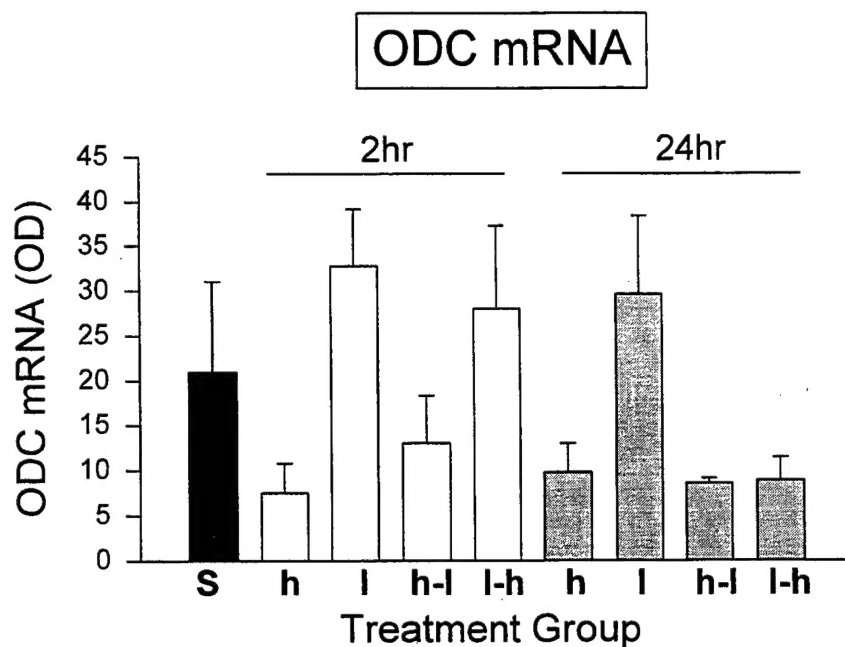
Amounts of iNOS mRNA expressed as mean optical density (OD) units  $\pm$  SEM as determined by densitometry. The four treatments are defined on the x-axis [HBO-only (h), ischaemia-only (I), HBO before ischaemia (h+I), and ischaemia before HBO (I+h)] and are grouped by the time of sacrifice (2 or 24 hours) separately from the sham (S).

2 hour					24 hour			
	h	I	h-I	I-h	h	I	h-I	I-h
S				*			*	*
h				*	n/a	n/a	n/a	
I				*	n/a		n/a	n/a
h			h-I	*	n/a	n/a		n/a
			I-h		n/a	n/a	n/a	
2					h			*
h						I		*
							h-I	*
								I-h
n= (3) (3) (3) (3) (4)					(3) (2) (3) (4)			

**TABLE 2. Statistical Analysis Summary Table for iNOS mRNA**

Sample size (n) is listed below each group; asterisk (\*) denotes  $p < 0.05$ ; n/a is used where contrasts were not made.





**FIGURE 2.**

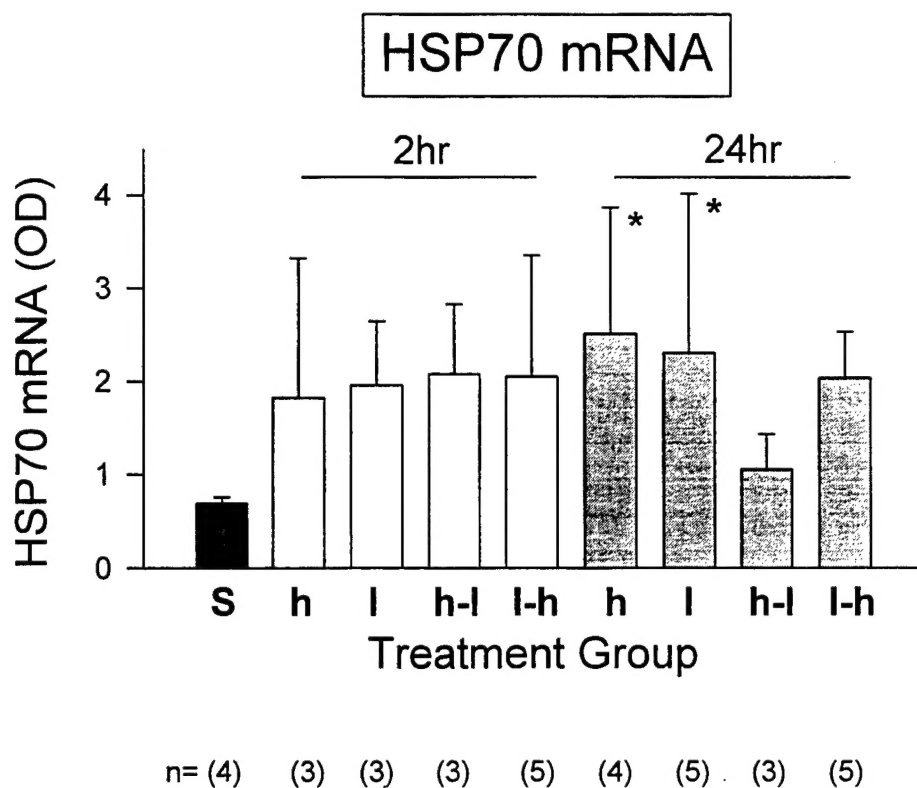
**Effects of HBO and/or ischaemia-reperfusion on ODC mRNA Expression**

Amounts of ODC mRNA expressed as mean optical density (OD) units  $\pm$  SEM as determined by densitometry. The four treatments are defined on the x-axis [HBO-only (h), ischaemia-only (l), HBO before ischaemia (h+l), and ischaemia before HBO (l+h)] and are grouped by the time of sacrifice (2 or 24 hours) separately from the sham (S).

2 hour					24 hour			
	h	l	h-l	l-h	h	l	h-l	l-h
S	*	*			*	*	*	*
h	h	*		*		n/a	n/a	n/a
		l	*		n/a		n/a	n/a
			h-l	*	n/a	n/a		n/a
				l-h	n/a	n/a	n/a	*
24h	h	*			h	*		
		l	*	*		l	*	*
			h-l				h-l	
				l-h				l-h
n= (4) (3) (4) (3) (4)					(5) (5) (3) (5)			

**TABLE 3. Statistical Analysis Summary Table for ODC mRNA**

Sample size (n) is listed below each group; asterisk (\*) denotes  $p < 0.05$ ; n/a is used where contrasts were not made.



**FIGURE 3.**

**Effects of HBO and/or ischaemia-reperfusion on HSP70 mRNA Expression**

Amounts of HSP70 mRNA expressed as mean optical density (OD) units  $\pm$  SEM as determined by densitometry. The four treatments are defined on the x-axis [HBO-only (h), ischaemia-only (I), HBO before ischaemia (h+I), and ischaemia before HBO (I+h)] and are grouped by the time of sacrifice (2 or 24 hours) separately from the sham (S). Only the groups that are significantly different ( $p < 0.05$ ) from the sham are denoted by an asterisk (\*). Group sample sizes (n) are listed below each group.

**IV. CONCLUSIONS**

Given that our analysis did not include time points between 2 and 24hrs, we are unable to make any conclusions relating to fluctuation in iNOS expression within that period. The absence of HBO's ability to have decreased the levels of iNOS expression at the 24hr time period does not help us discern whether HBO has the potential to ameliorate the potential damage by NO or subsequent reactive species induced by ischaemia-reperfusion injury. Further analysis of reactive oxygen or radical species in this experimental scenario is important to clear up this dilemma. The information concerning HSP70 mRNA expression in these experiments is equivocal. The ODC mRNA response is consistent with previously reported

data. The decrease in ODC due to HBO with or without ischaemia has not been previously reported. From this data, one promising role for HBO in countering the destructive effects of ischaemia-reperfusion lies not in the decreasing of iNOS expression, but rather in the apparent suppression of ODC expression. Whereby, the production of polyamines is impeded, decreasing their overall contribution to inflammation and tissue edema.

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